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**RESEARCH ARTICLE**

# Calcium dobesilate attenuates vascular injury and the progression of diabetic retinopathy in streptozotocin-induced diabetic rats

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## Abstract

**Background** Diabetic retinopathy (DR) is a highly specific vascular complication of type 1 and type 2 diabetes mellitus. Calcium dobesilate (DOBE) has been tested in the treatment of diabetic retinopathy showing a slowdown of the progression of the disease after long-term oral treatment. The aim of this study was to determine the effects of DOBE on vascular and diabetic retinopathy in streptozotocin (STZ) diabetic rats.

**Methods** Diabetes was induced in wistar rats by the administration of STZ (60 mg/kg, i.p.). Rats were divided into three groups ($n = 30$). Group 0 (GO): nondiabetic rats. Group 1 (G1): 14 months of insulin treatment after diabetes development. Group 2 (G2): 14 months of insulin treatment after diabetes development plus DOBE (500 mg/kg/day). At the end of the treatment, vascular reactivity was tested. The study of the vascularization of the retina was performed on wholemounts of trypsin retinal digest preparations and retinal sections.

**Results** Relaxation induced by acetylcholine decreased in the aorta arteries from diabetic rats but it was restored to control values in the DOBE-treated group ($71.8 \pm 4.5\%$, $53.3 \pm 0.5\%$, $67.4 \pm 4.6\%$ in group 0, 1 and 2 respectively). DOBE treatment also restored noradrenaline ($1.08 \pm 0.05$ g, $1.70 \pm 0.08$ g, $1.13 \pm 0.05$ g in group 0, 1 and 2 respectively) and caffeine-induced contractions. Diabetic state did not cause any alteration in mesenteric arteries. The analysis of the retinal digests showed vascular tortuosity, acellular capillaries, focal accumulations of capillaries and reduction of the number of pericytes in G1. The vascular changes observed in G2 seem to be intermediate between the control and the diabetic rats.

**Conclusions** We showed that long-term treatment with DOBE attenuated the progression of diabetic retinopathy and the alterations in vascular reactivity in streptozotocin-induced diabetic rats. Copyright © 2005 John Wiley & Sons, Ltd.

**Keywords** pericytes; endothelial cells; vascular reactivity; microangiopathy; rats; diabetic retinopathy

## Introduction

Diabetic retinopathy (DR) is a highly specific vascular complication of type 1 and type 2 diabetes mellitus [1]. The process begins in isolated capillaries that become acellular and nonperfused, extends to groups of capillaries and

then advances to involve the arterioles and their side branches. Loss of pericytes and endothelial cells from the capillary bed, endothelial cell proliferation and the development of tortuous swollen vessels are well-known features in the histology of diabetic retinopathy [2–4]. The most widely accepted animal model of diabetic retinopathy is the streptozotocin (STZ) model. The observation that streptozotocin-induced diabetic rats developed retinal lesions similar to those observed in humans with diabetes has attracted widespread attention to this animal model of human diabetic retinopathy [5].

Vascular injury and cardiovascular dysfunction are the leading causes of morbidity and mortality in diabetic patients [6,7], yet the underlying mechanisms remain unclear. Impaired endothelium-dependent relaxation has been described in human and in animal models [8–10]. A variety of mechanisms have been proposed to explain this observation, and the role of nitric oxide (NO), in particular, has been evaluated. Also, endothelial dysfunction following hyperglycemia is associated with increased generation of oxygen free radicals and vasoconstrictors [11].

In the study 'Diabetes Control and Complication Trial' (DCCT) [12], a definitive relationship was demonstrated in type 1 diabetes between hyperglycemia and diabetic microvascular complications, including diabetic retinopathy. The DCCT showed that even an reasonable control of glycemia in diabetic patients will not fully prevent the development of diabetic retinopathy. For that reason, a concomitant treatment with some other drugs that prevent or delay the development of micro- and macrovascular complications in diabetes seems to be necessary.

Calcium dobesilate (2,5-Dihydroxybenzenesulfonic acid calcium salt) has been tested in the treatment of diabetic retinopathy. Controversies arise regarding the benefits of DOBE treatment in DR; although clinical studies show a slowdown in the progression of the disease after long-term oral treatment, others have shown no beneficial effects of DOBE [13–15]. The main effect of the drug is to reduce microvascular permeability [16], leading to improved visual acuity. The pharmacological activity may be explained in part by the antioxidant properties of DOBE [17,18], through its action on the endothelium, which increases NO synthesis [15], and endothelium-dependent relaxation [18]. On the other hand, there is a disagreement about the possible effect of diabetes mellitus on vasoconstriction [19,20].

To the best of our knowledge, there are no histological studies that demonstrate the effect of DOBE on retinal microcirculation and the cells involved in the regulation of these vessels. Given these premises, the aim of this work was to determine the pharmacological benefits of long-term treatment with DOBE on vascular alterations and histological changes in the retinal vasculature associated with DR in STZ diabetic rats.

# Materials and methods

## Animal and experimental diabetes

Three groups ($n = 30$) of male Wistar rats weighing $253 \pm 5.1$ g were used at the beginning of the study. Rats were housed separately in cages in an air-conditioned room with a 12-h light/dark cycle. Rats were fed standard rat chow and allowed access to water *ad libitum*. Diabetes was induced by a single intraperitoneal injection of streptozotocin 60 mg/kg of body weight in citrate buffer (0.1 M) with a pH of 4.5. After 72 h, tail blood samples were obtained and glucose concentration was measured with a glucometer Accutrend (Boehringer Mannheim, Mannheim, Germany). Diabetes induction was considered successful when glycemia was higher than 20 mmol/L. Those rats that did not achieve this blood level of glucose were removed from the study. Age- and sex-matched nondiabetic control animals (**G0**) ($n = 10$) received an injection of the same volume of citrate buffer. In diabetic rats, a sustained-release bovine insulin pellet (Limplat©, Sustained release Insulin implant) was implanted subcutaneously 72 h after STZ injection and replaced every 45 days. These implants contained palmitic acid as an excipient and were implanted without

**Table 1. Blood glucose levels and body weight**

| | Group 0 | | Group 1 | | Group 2 | |
|---|---|---|---|---|---|---|
| Time | Glucose | Body weight | Glucose | Body weight | Glucose | Body weight |
| Day 1 | $83.5 \pm 3.5$ | $253 \pm 5$ | $94.3 \pm 11.5$ | $249 \pm 1.5$ | $84.0 \pm 6.0$ | $261 \pm 7$ |
| Day 0 | $83.5 \pm 0.5$ | $257 \pm 6$ | $94.7 \pm 6.0$ | $245 \pm 9.8$ | $80.5 \pm 0.5$ | $260 \pm 5$ |
| Day 2 | $88.6 \pm 4.4$ | $249 \pm 2$ | $486.6 \pm 13.3^{***}$ | $239 \pm 16$ | $485.4 \pm 15.0^{***}$ | $283 \pm 22$ |
| 1 month | $87.6 \pm 12.1$ | $336 \pm 6$ | $117.3 \pm 15.1$ | $299 \pm 12$ | $138.3 \pm 10.8$ | $344 \pm 25$ |
| 3 month | $86.4 \pm 11.8$ | $438 \pm 13$ | $175.3 \pm 26.6^{*}$ | $325 \pm 14$ | $161.6 \pm 10.0$ | $388 \pm 20$ |
| 5 month | $85.2 \pm 11.7$ | $462 \pm 11$ | $167.6 \pm 12.4^{*}$ | $350 \pm 26$ | $180.1 \pm 10.0^{**}$ | $392 \pm 17$ |
| 7 month | $86.4 \pm 10.1$ | $423 \pm 34$ | $113.6 \pm 24.2$ | $379 \pm 28$ | $181.7 \pm 13.5^{**}$ | $419 \pm 11$ |
| 9 month | $85.2 \pm 11.1$ | $420 \pm 20$ | $115.1 \pm 10.0$ | $389 \pm 27$ | $159.3 \pm 10.2$ | $412 \pm 19$ |
| 11 month | $89.6 \pm 9.6$ | $440 \pm 17$ | $210.8 \pm 12.2^{**}$ | $400 \pm 12$ | $180.6 \pm 11.4^{**}$ | $420 \pm 12$ |
| 13 month | $93.4 \pm 3.9$ | $460 \pm 10$ | $215.7 \pm 11.4^{**}$ | $405 \pm 13$ | $196.4 \pm 20.5^{**}$ | $425 \pm 14$ |
| 14 month | $97.7 \pm 6.5$ | $441 \pm 7$ | $222.4^{**} \pm 16.8^{**}$ | $400 \pm 13$ | $206.7 \pm 15.6^{**}$ | $415 \pm 17$ |

Values of blood glucose and body weight throughout the experiment. Day 0 is considered when streptozotocin was injected. Glucose levels are given in mg/100 mL and body weight in grams. Values denote the mean $\pm$ s.e. of mean of $n = 6$ to 8 animals per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, respect to control (Group 0) at the corresponding time point.

sutures under the dorsal skin of rats slightly anesthetized with ethyl ether. Every implant gradually released the insulin at a dose of 2 units per day. Diabetic rats were randomized into a group treated with insulin alone (**G1**) ($n = 10$) and a group treated with insulin plus DOBE (DOBE was dissolved in water and administered orally with a esophageal tube at a dose of 500 mg/kg/day) (**G2**) ($n = 10$). Data on body weight and glycemia were monitored throughout the study and are shown in Table 1.

The animals were anesthetized with ethyl ether and killed by exsanguination from the common carotid in the 14th month of the experiment. The thoracic aorta and mesenteric artery (4th branch, approximately 175 μm inside diameter) were rapidly removed and placed in Krebs-Henseleit solution (KHS) having the following composition (mM): NaCl 119, KCl 4.7, $NaHCO_3$ 25, $MgSO_4$ 1.0, glucose 11.1; $KH_2PO_4$ 1.2 and $CaCl_2$ 2.5. Care and use of animals conformed to the ARVO Statement for the use of animals in ophthalmic and vision research.

## Aortic rings

Adherent fat and surrounding tissue were cleaned off and the arteries were cut into rings approximately 2- to 3-mm wide. The rings were then suspended between two stainless steel hooks in organ baths containing 10 mL of Krebs-Henseleit solution. The solution was kept at $36 \pm 0.5$ °C and gassed continuously with a 95% $O_2$-5% $CO_2$ gas mixture. Aortic rings were mounted under 1 g of resting tension. Each preparation was allowed to equilibrate for 60 min. Contractile responses were measured isometrically by means of force-displacement transducers (Grass FT 03) and were recorded on a Grass polygraph as described previously [21]. The isometric force was also digitalized by a MacLab A/D converter (Chart v3.2, A.D Instruments Pty. Ltd., Castle Hill, Australia) and stored and displayed on a Mackintosh computer [22].

## Mesenteric arteries

Using a dissecting microscope, a segment of small mesenteric artery of the rat, approximately 2 mm in length, corresponding to a fourth order branch of the superior mesenteric artery, was carefully dissected under a light microscope, free of connective tissues and its vein. The artery was mounted in a small vessel myograph (Multy Myograph system 610M) [23] using 40 μm tungsten wires (Westinghouse Inc.). The vessel was set to a tension equivalent to that generated at 0.9 times the diameter of the vessel at 100 mmHg transmural pressure [24]. Vessels were allowed to equilibrate at $37 \pm 0.5$ °C and gassed continuously with a 95% $O_2$-5% $CO_2$ gas mixture. The isometric force was also digitalized by Myodaq 2.01 program (Danish Myo Technology, Denmark) and stored and displayed on a personal computer.

## Retinal vascular digest

Eyes were extracted immediately after death and fixed in paraformaldehyde 4% in phosphate buffered saline (PBS) 0.1 M, pH 7.4 at 4 °C for two days and then processed to obtain isolated preparations of the vascular network by trypsin digestion, as follows.

The retinas were first removed from the eyecup and then sectioned with four equidistant cuts for the preparation of retinal flat mounts. The tissues were then immersed and rinsed overnight in a phosphate buffered saline (PBS) and then all but the vascular elements of the retinas were digested using mild enzyme incubations in a modified form of the methods described by Kuwabara and Cogan [25]. The retinas were incubated for one hour in 3% trypsin [(Seromed 1 : 250) Biochrom KG, Berlin, Germany)] in 0.1 M Tris buffer (pH 7.8) at 37 °C. with gentle agitation. Then, the retinas were placed in PBS, the vitreous humor was removed and the neural retinal tissue was partially eliminated by delicate stroking with a thin paintbrush. Next, the retinas were incubated in 3% trypsin in 0.1 M Tris buffer (pH 7.8) at 37 °C by gentle agitation for a further 15 min until isolated preparations of the retinal vascular network were obtained. The resulting vascular tissues were then washed in distilled water and transferred, floating in distilled water, to a slide for appropriate spreading of the vascular tissue. The distilled water was gently removed with a Pasteur micropipette to avoid tissue distortion. Finally, the retinal vasculature was stained with periodic acid-Schiff and counterstained with hematoxylin.

## Experimental procedure

### 1. Vascular reactivity

After the equilibration period, a contraction was induced in mesenteric or aortic rings with a single concentration of KCl (80 mM) or noradrenaline (NA) ($10^{-6}$ M in aortic and $10^{-5}$ M in mesenteric rings). Endothelium-dependent (acetylcholine, $10^{-8}$–$10^{-4}$ M) and endothelium-independent responses (sodium nitroprusside, $10^{-8}$–$10^{-4}$ M) and ß-adrenergic-dependent relaxation (isoproterenol, $10^{-8}$–$10^{-4}$ M, only in aorta) were performed in NA-precontracted arteries.

The experimental protocol by Itho *et al*. [26] was used to test the possible effects of DOBE on caffeine-induced intracellular $Ca^{2+}$ release. It is known that caffeine depletes caffeine-/ryanodine-sensitive $Ca^{2+}$ pools, and not all intracellular calcium pools, but we have used this technique as a rough measure. In all the groups studied, the amount of $Ca^{2+}$ stored in the aortic rings was estimated from the size of the contractions evoked by 20 mM caffeine after incubation in $0Ca^{2+}$-KHS plus EGTA (2 mM).

### 2. Diabetic retinopathy

The retinal study was split into two phases to assess the effect of DOBE on diabetic retinopathy. The first phase

quantified the number of endothelial cells and pericytes. The second phase quantified the retinal area occupied by the vessel.

To determine the number of pericytes and endothelial cells in the three experimental groups, different retinal areas were chosen at low magnification, the only criteria being that the entire area be free of nonvascular elements, be well stained, and be evenly spread to permit unobstructed viewing of all capillaries within the region.

Morphological criteria were used to differentiate the pericytes from the endothelial cells. Thus, endothelial cells were oriented along the axis of the capillary and had long pale-staining nuclei, whereas the pericyte bulged in the wall of the capillary, and had a small, round, dark-staining nucleus (Figure 1A).

The number of pericytes and endothelial cells was quantified following a masking procedure. Photomicrographs of seven zones from each retina were taken at random discarding those areas in which digestion of the retinal parenchyma was incomplete or in which vessels were not visible throughout. Photographs were taken with a light microscope (Jenalumar, Carl Zeiss Jena) using 50 × 0.8 magnification; this gave rectangular zones 266.6666 µm long × 172.2222 µm wide covering a retinal area of 0.0459 $mm^2$.

The retinal area occupied by the vessels was studied by a computer-assisted morphometric analysis [27,28]. We used the Metamorph Imaging System version 4.5 computer program (© Universal Imaging Corps) in association with an Axioplan 2 Imaging Microscope (Zeiss). The entire retinal wholemount was photographed using the motorized stage of the microscope to scan the whole preparation along the x-y axis. Thus, all subsequent photographed fields were contiguous and were taken systematically to assure that no portion of the retinal whole mount would be omitted or duplicated. Photographs were taken with a 20× microscope lens, giving an area of 0.1889 $mm^2$ per photograph. Photographs showing the edge of the wholemount and any images in which digestion of the retinal parenchyma was incomplete or in which vessels were not visible throughout, were discarded. The resulting images were processed with the Threshold Tool included in the Metamorph Imaging System. This tool can be used to create a boundary on the basis of the image's gray scale or color levels. Areas of the image that are marked with the threes holding overlay (in this study retinal vessels) were included in the measurement and processing (Figure 1B,C). Two of the eight retinas from G0 rats were discarded because of poor quality. A total of 904 vascular areas were studied in this way (Table 2).

## Drugs

Drugs were purchased from Sigma (Madrid, Spain) unless otherwise stated. DOBE (2,5-Dihydroxybenzenesulfonic acid calcium salt) was donated by the Esteve Foundation. The concentrations refer as the final molar concentration in the organ chamber solution. Ascorbic acid was added

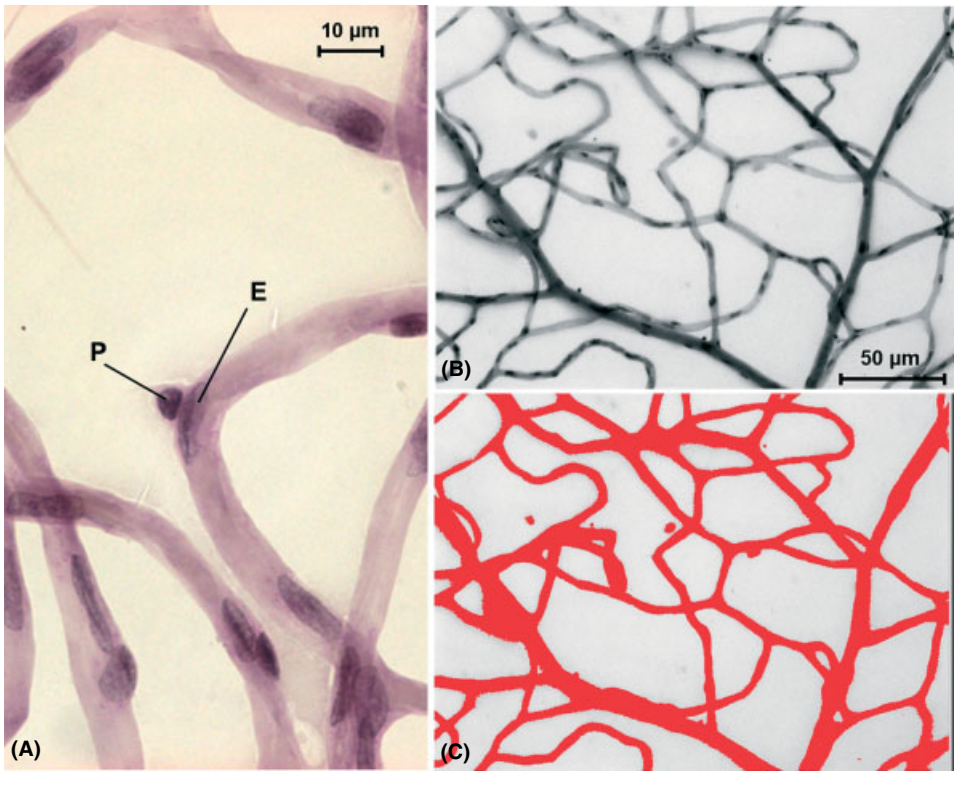


**Figure 1. Optical images of trypsin-digested retinas isolated from rats in G0 (control, nondiabetic). (A) E: endothelial cells; P: pericytes, Bar: 10 µm. (B,C) The zones of the image that were marked with the thresholding overlay (retinal vessels in red) were included in the measurement and processing, Bar: 50 µm**

**Table 2. Measurements of retinal vessels**

| Group | Rat | Selected microscopic fields[a] | Total retinal area measured[b] ($mm^2$) | Total vascular area measured[c] ($mm^2$) | Capillary density[d] (%) |
|---|---|---|---|---|---|
| G0 | 1 | 92 | 17.38 | 7.55 | 43.90 |
| | 2 | 79 | 14.93 | 6.52 | 44.23 |
| | 3 | 34 | 6.42 | 2.88 | 46.23 |
| | 4 | 33 | 6.24 | 2.45 | 40.50 |
| | 5 | 77 | 14.55 | 6.12 | 42.62 |
| | 6 | 57 | 10.77 | 4.53 | 43.60 |
| G1 | 1 | 40 | 7.56 | 3.53 | 46.69 |
| | 2 | 70 | 13.23 | 6.47 | 49.62 |
| | 3 | 90 | 17.01 | 9.18 | 54.58 |
| | 4 | 106 | 20.03 | 10.06 | 50.71 |
| G2 | 1 | 95 | 17.95 | 8.29 | 46.68 |
| | 2 | 88 | 16.63 | 6.40 | 38.93 |
| | 3 | 15 | 2.83 | 1.15 | 43.56 |
| | 4 | 29 | 5.48 | 2.78 | 52.55 |

[a]Photographs remaining after discarding those judged unsuitable.
[b]Total retinal area measured per rat.
[c]The total area per animal occupied by the vessels within the retinal area measured.
[d]The percentage of the measured retinal area occupied by vascular area.

to each daily prepared solution of noradrenaline to avoid noradrenaline oxidation. Working solutions were made in Krebs-Henseleit solution.

## Statistical analyses

In the experiments with arteries, all values represent mean ± s.e. mean of 10 rats in each group of experiments. The different groups were compared by two way ANOVA test or Student's T test and differences were considered significant when $P < 0.05$.

The data from the analysis of the retina vascular digestions were input to the SPSS 11.5 (comprehensive statistical software; SPSS Inc©). Statistical analysis of pericyte counting was performed by using the ANOVA test after confirming that the variations were homogeneous. In the analysis of the vascular areas, the conditions required for the use of ANOVA test were not fulfilled. Thus, they were studied with the Kruskal-Wallis test. Each animal was considered a variable for analysis. Differences were considered significant when $P < 0.05$.

# Results

## Experimental diabetes

Body weight and plasma glucose levels were monitored throughout the study and are shown in Table 1. Forty-eight hours after streptozotocin administration, rats were diabetic with glucose levels above 400 mg/100 mL. After the administration of the insulin implant, glucose levels in groups 1 and 2 decreased and it were maintained at levels of approximately 150 to 200 mg/mL. This range of glucose is considered normal in the rat. Body weight did not change with respect to control in any of the groups studied.

## Vasoconstriction studies

In the first group of experiments, we studied the contractile responses induced by NA $10^{-6}$ M and $10^{-5}$ M in aorta and mesenteric arteries respectively. As shown in Figure 2, the contraction induced by NA in aortic rings was significantly increased in STZ-diabetic rats (G1) when compared with age-matched controls (G0) ($1.70 \pm 0.08$ g vs $1.08 \pm 0.05$ g, $n = 10$, $P < 0.01$ in diabetic and in control rats respectively) but it was restored to control levels in DOBE-treated animals (G2) ($1.13 \pm 0.05$ g vs $1.08 \pm 0.05$ g, DOBE-treated animals and control animals respectively). When mesenteric arteries were contracted with noradrenaline, we found no significant differences in any of the groups studied.

High $K^+$-induced contractions in aortic rings were similar in all the groups studied, whereas, in mesenteric arteries, the contractions induced by KCl 80 mM decreased in the DOBE-treated group (Figure 2).

## Agonist-induced relaxation

### Endothelium-dependent and independent relaxations

In both aorta and mesenteric rings, ACh caused a concentration-dependent relaxation in vessels precontracted with NA isolated from the different groups studied. The diabetic state (G1) induced a decrease in the endothelium-dependent relaxation in the aorta but not in the mesenteric artery. Treatment with DOBE (500 mg/kg/day, G2) restored the endothelium-dependent relaxation in aortic rings to normal. The maximal relaxations were $71.8 \pm 4.5\%$ vs $53.3 \pm 0.5\%$ ($P < 0.05$, $n = 10$) in the control and diabetic groups respectively (G0 and G1) and $67.4 \pm 4.6\%$ in DOBE-treated rats (G2) (Figure 3). There were no differences in terms of endothelium-dependent relaxation in mesenteric arteries among all the groups studied (Figure 3). Endothelium-independent relaxation induced by SNP was similar in all the groups studied, in both arteries (Figure 3).

### Relaxation induced by isoproterenol

In aortic rings precontracted with NA, relaxation was induced by isoproterenol ($10^{-8}$–$10^{-4}$ M) to test changes in $\beta$-adrenoreceptor induced by the diabetic state. Comparison of the control group and the diabetic group shows that, although the maximal response to isoproterenol ($10^{-4}$ M) was similar in both groups, the concentration response curve was shifted slightly to the right in the diabetic group (G1). ANOVA showed statistically significant differences between the two curves. Vessels from DOBE-treated animals showed a relaxation induced by isoproterenol midway between

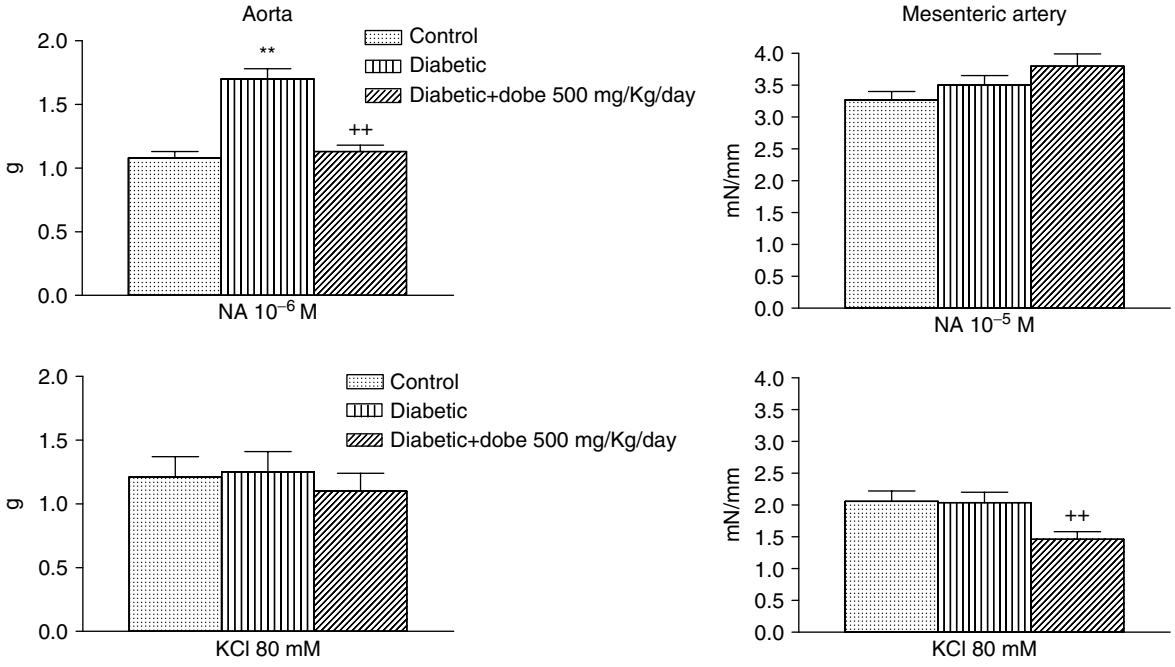


**Figure 2. Effect of DOBE treatment on contractions induced by NA $10^{-6}$ M or KCl 80 mM in either aorta (left panels) or mesenteric (right panels) arteries isolated from the different groups studied. Each bar shows the mean ± s.e. mean ($n = 10$, $^{**}P < 0.01$ with respect to the control group; $^{++}P < 0.01$ with respect to the diabetic group, G1)**

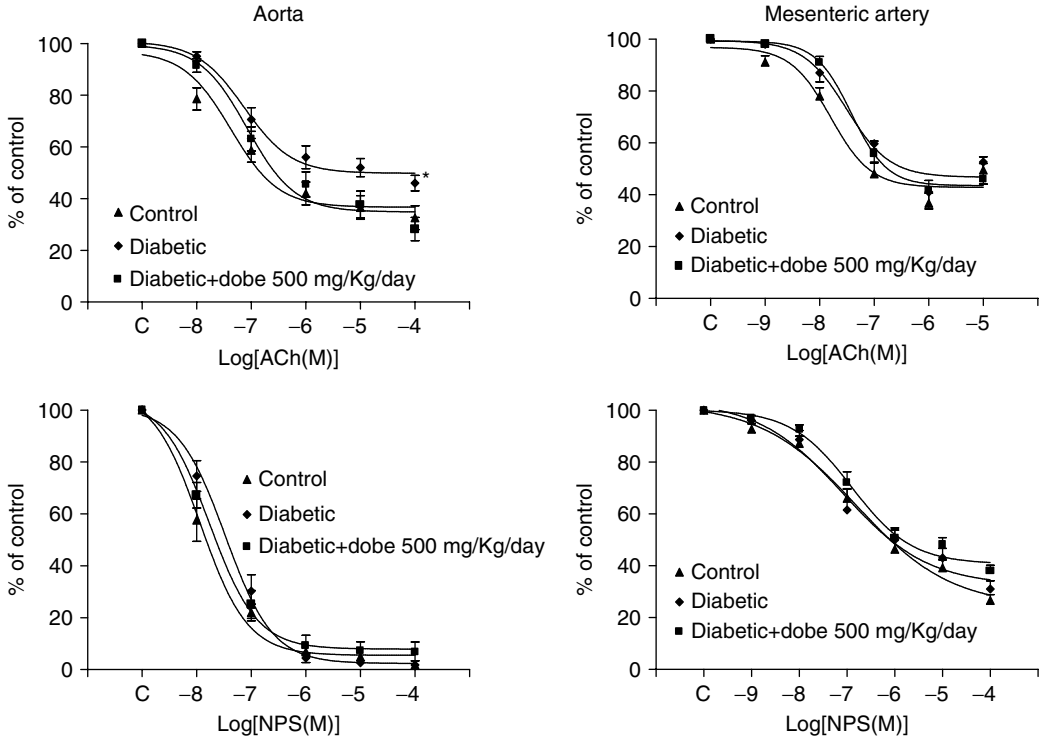


**Figure 3. Effect of DOBE treatment on endothelium-dependent relaxation induced by ACh ($10^{-8}$–$10^{-4}$ M) (upper panels) and endothelium-independent relaxations induced by sodium nitroprusside ($10^{-8}$–$10^{-4}$ M) (lower panels) in either aorta or mesenteric arteries isolated from the different groups studied. Each point shows the mean ± s.e. mean ($n = 10$, $^{*}P < 0.05$ with respect to the control group G0)**

control and diabetic animals, although this effect was not statistically significant (Figure 4).

### Contraction induced by caffeine

To study if there was an increase in the amount of calcium from intracellular pools in diabetic rats, contractions induced by caffeine (20 mM) in $0Ca^{2+}$ medium were induced in aortic rings. As shown in Figure 5, the contractions induced by caffeine were $0.10 \pm 0.02$ g in the control group (G0); $0.22 \pm 0.03$ g ($P < 0.001$ with respect to the control, G0) in diabetic rats (G1) and $0.16 \pm 0.03$ g ($P < 0.05$ with respect to G0) in diabetic rats treated with DOBE (G2).

## Retinal vascular digest

### Morphological findings

In control rats, the size of retinal vessels was regular throughout and no dilation or narrowing was detected in the capillary lumen (Figure 6A). However, in diabetic rats (G1), microvascular lesions consistent with the early stage of diabetic retinopathy were detected clearly (Figure 6B,7). We found: (1) vascular tortuosity (Figure 6B,7A); (2) numerous areas of focal accumulations of capillaries (Figure 6B,7D); (3) acellular capillaries (Figure 7C); (4) abundant ghost pericytes (Figure 7B). However, no microaneurysms were found. In age-matched diabetic rats treated with DOBE (G2), we found areas of tortuous vessels (Figure 6C) with some acellular capillaries and ghost pericytes. However, there were less microvascular lesions in the retina than in G1 rats (Figure 6C). Areas of focal capillary accumulation were generally scarcer than in the control group (G0) (Figure 6C).

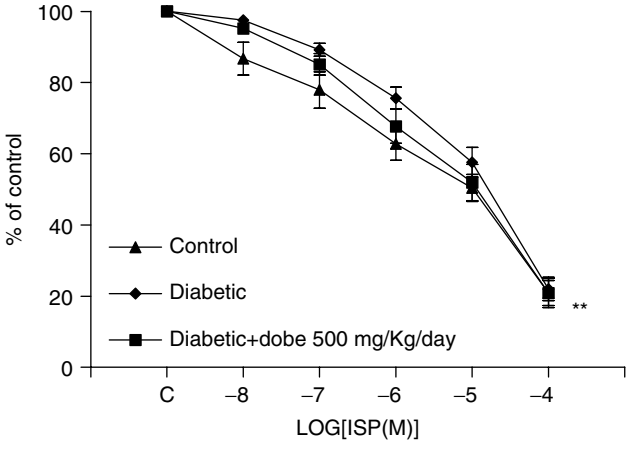


**Figure 4. Effect of DOBE treatment on relaxation induced by isoproterenol ($10^{-8}$–$10^{-4}$ M) in aorta isolated from the different groups studied. Each point shows the mean ± s.e. mean ($n = 10$, $^{**}P < 0.01$ with respect to the control group G0)**

### Cell counting

Variations in the number of perycites and endothelial cells, as a typical feature of DR, were quantified. All three groups differed significantly from one another in the number of perycites (Table 3). Intergroup comparisons revealed that diabetic rats (G1 and G2) had a significant lesser number of perycites than controls rats ($P < 0.000$ in both instances). The difference being established by 95% confidence interval of 12.74; 20.58 for G0 versus G1 and 4.91; 12.76 for G0 versus G2. Additionally, diabetic rats treated with insulin alone exhibited significantly less pericytes than diabetic rats treated with DOBE ($P < 001$). The difference was determined by a 95% confidence interval of 3.29;12.34.

The mean endothelial cell count in G2 rats (insulin plus DOBE) was midway between the other groups (G0 and G1). However, the differences between groups were not statistically significant (Table 3).

### Vascular areas

Values for retinas of rats treated with DOBE (G2) were midway between those of the control rats and the rats treated with insulin alone (G1). The differences among the three groups were not statistically significant (Table 4).

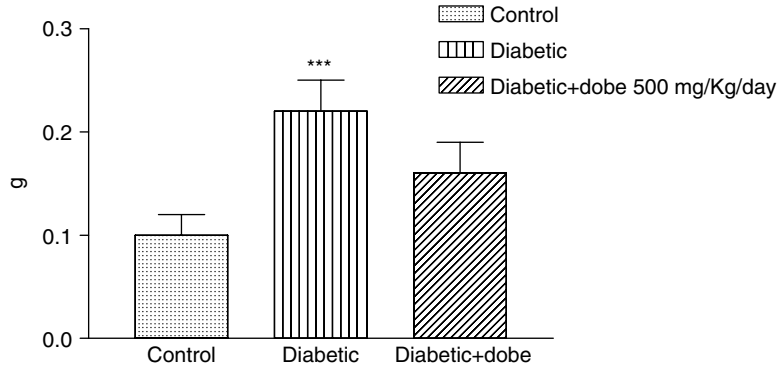


**Figure 5. Effect of DOBE on contraction induced by caffeine (20 mM) in a $Ca^{2+}$-free medium in aortic rings from the different groups studied. Each bar shows the mean ± s.e. mean ($n = 10$, $^{***}P < 0.001$ with respect to the control group G0; $^{++}P < 0.01$ with respect to the diabetic group G1)**

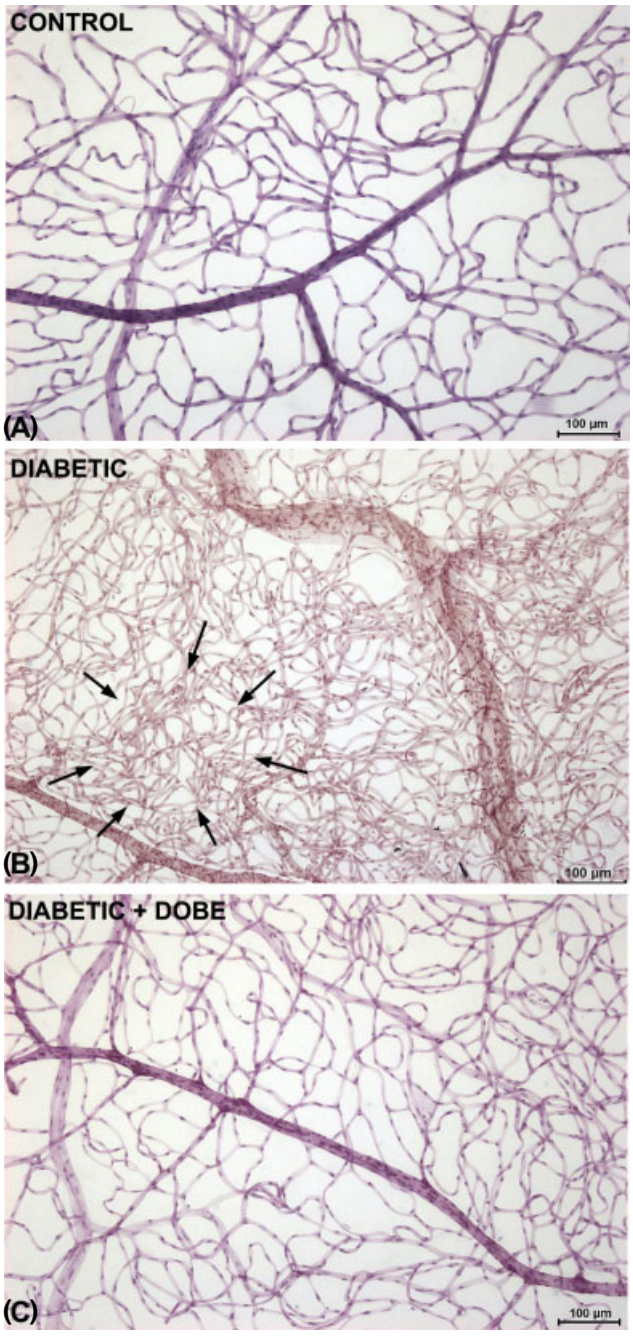


**Figure 6. Comparison of optical images of trypsin-digested retinas isolated from rats. (A) In G0 (control, nondiabetic rats), none of the characteristics of diabetic microangiopathy were observed. (B) In G1 (streptozotocin-induced diabetic rats treated with insulin alone), areas of tortuous vessels and focal accumulation of capillaries (arrows) are shown. (C) In G2 (streptozotocin-induced diabetic rats treated with insulin plus DOBE), a lower presence of focal accumulation of capillaries than in samples from G1 is observed. Bar: 100 µm**

**Table 3. Counts of pericytes and endothelial cells in the three groups studied. Average number of cells in an area of 0.0459 mm$^2$**

| | Groups | Samples | Means | Standard deviation | *P* |
|---|---|---|---|---|---|
| Pericytes | G0 | 8 | 30.87 | ±2.15 | 0.000[a] |
| | G1 | 4 | 14.21 | ±2.98 | |
| | G2 | 4 | 22.03 | ±1.94 | |
| Endothelial cells | G0 | 8 | 43.49 | ±4.32 | 0.58[b] |
| | G1 | 4 | 48.64 | ±12.75 | |
| | G2 | 4 | 40.71 | ±3.31 | |
| Endothelial cells/pericytes | G0 | 8 | 1.41 | ±0.067 | 0.002 |
| | G1 | 4 | 3.82 | ±1.94 | |
| | G2 | 4 | 1.88 | ±0.12 | |

G0, control rats; G1, diabetic rats plus insulin alone; G2, diabetic rats plus insulin and DOBE.
[a]ANOVA.
[b]Kruskal–Wallis test.

**Table 4. Mean vascular areas and standard deviations**

| Groups | N* | Selected microscopic fields[1]* | Means (µm$^2$) | Standard deviation | *P** |
|---|---|---|---|---|---|
| G0 | 6 | 372 | 80660.91 | ±3650.80 | 0.051 |
| G1 | 4 | 306 | 94358.28 | ±5772.89 | |
| G2 | 4 | 227 | 83163.27 | ±10577.54 | |

N*: number of rats analyzed; 1: Each microscopic field *correspond to 0.1889 mm$^2$; G0, control; G1, diabetic rats plus insulin alone; G2, diabetic rats plus insulin and DOBE; *Kruskal–Wallis test. *Data correspond to the number of rats and selected microscopic fields shown in Table 2.

## Discussion

Epidemiological studies show that patients suffering from diabetes mellitus are particularly prone to cardiovascular disorders [29], such as macrovascular and microvascular alterations. The model of rats treated with STZ displays many of the features seen in human subjects with uncontrolled diabetes, including hyperglycemia, polydipsia, polyuria and weight loss [30]. However, there is disagreement about the possible effect of Diabetes Mellitus (DM) on vasoconstriction. Some authors have reported an attenuated maximal contractile response to noradrenaline, phenylephrine and methoxamine in aortae from STZ-treated rats [20], but no changes in sensitivity. In contrast, others have observed an increased maximal contractile response to noradrenaline and methoxamine [11,19]. We found, here, an increase in the NA-induced vasoconstriction in aortic rings but not in mesenteric rings. Interestingly, such vasoconstriction was considerably less in animals treated with insulin plus DOBE than in those treated with insulin alone. We hypothesized that this effect of DOBE might be due to the action on intracellular calcium and/or $\beta$-adrenoreceptors. In a previous study, our group reported that *in vitro* incubation with DOBE restored the increased contraction induced by NA in diabetic rats (BB/wor rats) to normal [31] and that this effect could be blocked by pretreating the aortic rings with propanolol, a $\beta$-receptor antagonist, indicating that DOBE might act at these receptors. However, from the present study, we cannot state a conclusion, since the effect of DOBE on isoproterenol-induced relaxation was not statistically significant. Some studies suggest that $\beta_2$-adrenoceptor-mediated vasorelaxation is, at least in part, mediated through NO release. Xu *et al*. [32] have shown that

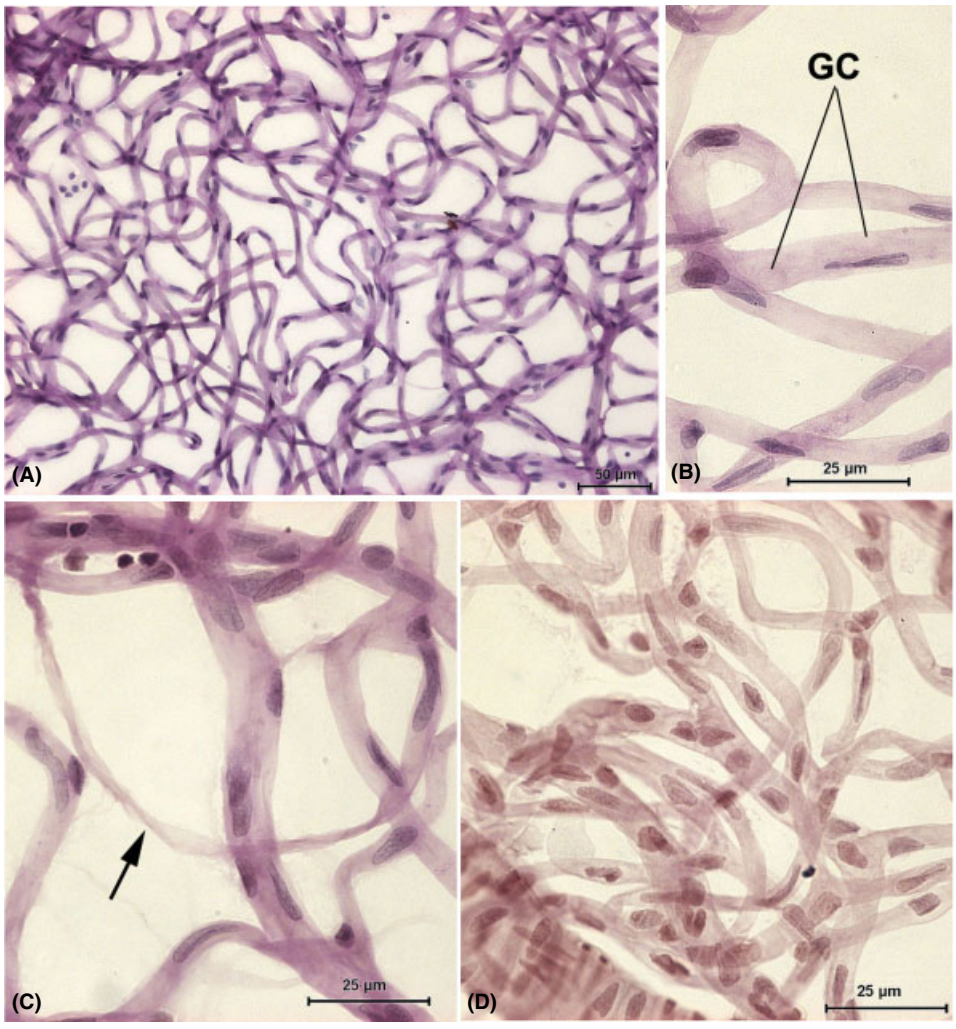


**Figure 7. Optical images of trypsin-digested retinas isolated from rats in G1 (streptozotocin-induced diabetic rats treated with insulin alone). General overview of the trypsin retinal digest showing areas of tortuous vessels and (A) focal accumulation of capillaries, Bar: 50 µm. (B) Ghost cell (GC), Bar: 25 µm. (C) Acellular capillary (arrow) Bar: 25 µm. (D) Magnification of focal accumulation of capillaries, Bar: 25 µm**

$\beta_2$-adrenergic stimulation and cAMP elevation activate the L-arginine/NO system in rabbit femoral arteries. The fact that the contraction induced by noradrenaline in diabetic rats is increased but the contraction induced by high $K^+$ did not suggest that the pool of intracellular calcium is enhanced in these rats with respect to the control rats. These data were confirmed by the results of the experiments with caffeine. Physiological and pharmacological evidence have indicated the presence of caffeine-sensitive intracellular pools that are distinct from the well-characterized inositol 1,4,5-triphosphate (IP3)-gated pools. We have studied the amount of calcium depleted by caffeine as a relative measure. Caffeine is used to deplete ryanodine-sensitive calcium pools [33,34] This is not a precise measurement, but since caffeine is used to measure the amount of intracellular calcium in all of the groups studied, and higher levels of calcium are obtained in one group, this indicates that there are more calcium in the intracellular stores of this group.

We found that the endothelium-dependent relaxation with ACh was attenuated in aortic rings but not in mesenteric arteries in G1 with respect to G0. Acetylcholine induced relaxation was already low even in control animals (G0) probably because the animals are old (16 months) and their relaxation does not reach the same level as in the younger animal. In preliminary reports, abnormal endothelium-dependent relaxation with acetylcholine has been described in some animal models [35,36] as well as in diabetic subjects [9,10,37]. Various explanations have been proposed, including a possible association of hyperglycemia with reduced endothelium-dependent relaxation: (1) the biological availability of NO may be reduced in diabetic states, since it is rapidly quenched by reactive oxygen radicals or by advanced glycation end-products [38,39]; (2) a role for glucose-stimulated increase in polyol pathway activity and (3) the redox changes associated with increased sorbitol flux [40,41]. The abnormal endothelium-dependent relaxation in aortae from diabetic rabbits has been restored to normal by superoxide-dismutase (SOD). This suggests a role for superoxide anions in the endothelial cell abnormality caused by diabetes mellitus

[42]. In addition, aortae from both normal and diabetic rabbits exposed to elevated concentrations of glucose *in vitro* demonstrate impairment of endothelium-dependent relaxation [43]. Therefore, the antioxidant properties of DOBE [17,18] and its action on NO synthesis [22] could together constitute an additional mechanism explaining the beneficial effect of DOBE in restoring endothelium-dependent relaxation in aortae from diabetic animals to normal.

On the other hand, we know that diabetes produces fundamental alterations of microcirculation and that the retina is one of the tissues that is most affected. In a general review of retinal vascular digestions, we found that diabetic animals treated with insulin alone (G1) developed the typical features associated with diabetic retinopathy, such as tortuous vessels with some acellular capillaries and focal accumulations of capillaries. However, in animals treated with insulin plus DOBE (G2), these complications seemed to be attenuated, as rats of this group presented apparently less severe vascular alterations.

The most striking change noted in our analysis of retinal microvascularization was in the number of pericytes. The pericytes in retinal tissue possess important contractile and vasoregulatory properties [44]. In our analysis of retinas, DOBE treatment ameliorates the loss of pericytes found in diabetic animals. Pericyte degeneration is one of the earliest histological changes observed in the development of diabetic retinopathy [2,4,32,45]. Pericyte death occurs mainly by apoptosis, which may be triggered by high glucose levels. Some antioxidant drugs have been shown to restore pericyte loss *in vivo* suggesting the involvement of oxidative injury during pericyte loss in diabetic retinopathy [46]. Supporting these findings, Li *et al*. [47] have shown that hyperglycemia followed by rapid glucose reduction can cause pericyte apoptosis mediated by oxidative stress. For that reason, we cannot rule out the antioxidant properties of DOBE [16–18,48] as one of the means by which the drug attenuates some of the features typical of diabetic retinopathy.

In the group of diabetic rats treated with insulin alone, the number of endothelial cells was higher than in the control group, but not significantly so. On the other hand, these cells were fewer in the group treated with DOBE than in the control group.

These findings are consistent with the analysis of the vascular areas, where the values recorded for rats treated with DOBE were closer to the values recorded in the control rats.

One of the reasons for diabetic progression of microangiopathy is the loss of endothelial cells with the appearance of acellular capillaries [44]. The increase in the number of endothelial cells in the diabetic group could be explained by the large decrease in the number of pericytes observed. It has been reported that the absence of pericytes provokes the stimulation of growth and division of endothelial cells. The pericytes exert an inhibitory influence on endothelial cell growth by the secretion of transforming growth factor-$\beta$1 (TGF-$\beta$1) [44]. Thus, the loss of pericytes could favor endothelial cell proliferation.

With DOBE treatment, the relaxation induced by acetylcholine, which decreased in the aorta of diabetic rats, was restored to normal. Noradrenaline- and caffeine-induced contractions, which increased in the aorta of diabetic rats, were also restored to normal. DOBE treatment further maintained pericyte numbers in the retinal capillaries; this may also promote proper functioning of the endothelial cells and hence an adequate vascular permeability.

In conclusion, the results show that early concomitant treatment with DOBE and insulin may counteract the deleterious changes associated with the onset of diabetic retinopathy.

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